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Behavioural Mechanisms of Sexual Isolation Involving Multiple Modalities and Their Inheritance

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Abstract

Speciation research dissects the genetics and evolution of reproductive barriers between parental species. Hybrids are the 'gatekeepers' of gene flow, so it is also important to understand the behavioural mechanisms and genetics of any potential isolation from their parental species. We tested the role of multiple behavioural barriers in reproductive isolation among closely related field crickets and their hybrids (*Teleogryllus oceanicus* and *T. commodus*). These species hybridise in the laboratory, but the behaviour of hybrids is unusual and there is little evidence for gene flow in the wild. We found that heterospecific pairs exhibited reduced rates of courtship behaviour due to discrimination by both sexes, and that this behavioural isolation was symmetrical. However, hybrids were not sexually selected against and exhibited high rates of courtship behaviour even though hybrid females are sterile. Using reciprocal hybrid crosses, we characterized patterns of interspecific divergence and inheritance in key sexual traits that might underlie the mating patterns we found: calling song, courtship song and cuticular hydrocarbons (CHCs). Song traits exhibited both sex-linkage and transgressive segregation, whereas CHCs exhibited only the latter. Calculations of the strength of isolation exerted by these sexual traits suggest that close-range signals are as important as long-distance signals in contributing to interspecific sexual isolation. The surprisingly weak mating barriers observed between hybrids and parental species highlights the need to examine reproductive isolating mechanisms and their genetic bases across different potential stages of introgressive hybridisation.

Keywords: acoustic signal, cuticular hydrocarbons, introgression, hybridization, multi-modal signalling, prezygotic isolation, reproductive isolation, sexual selection, speciation, *Teleogryllus*

Introduction

Multiple reproductive barriers are expected to evolve between incipient species, and such barriers often involve changes in the signals and preferences underlying mate recognition systems (Wells & Henry, 1998; Gleason & Ritchie, 1998; Mendelson & Shaw, 2005; Seehausen, 2006). When incipient species or those capable of hybridizing come into contact, hybrid individuals have therefore been suggested to be the key gatekeepers of

introgression, because their fate will determine the extent and direction of interspecific gene flow (Broyles, 2002; Borge *et al.*, 2005). Hybrids are generally expected to suffer reduced fitness compared to individuals of the parental species, due to intrinsic postzygotic factors such as physiological hybrid sterility and hybrid inviability, and extrinsic postzygotic factors such as disruptive ecological and sexual selection (Panhuis *et al.*, 2001; Servedio, 2004; Ritchie, 2007; Safran *et al.*, 2013; Scordato *et al.*, 2014). Hybrids may also be unfit because they exhibit aberrant courtship behaviours due to neurological or physiological defects, or intermediate behaviours that fall outside the range of the parental species' preferences (Naisbit *et al.*, 2001; Bridle *et al.* 2006). Sexual selection against such hybrids has numerous targets. Hybrids can be counter-selected due to their phenotypic distinctiveness (cichlid fish; Selz *et al.*, 2014), courtship failure (*Nasonia* wasps; Clark *et al.* 2014), intermediate sexual signals (*Chorthippus* grasshoppers; Bridle *et al.*, 2006), and other forms of behavioural sterility (*Heliconious* butterflies and *Schizocosa* wolf spiders; Naisbit *et al.*, 2001; Stratton & Uetz, 2006). Alternatively, hybrids can experience increased reproductive success due to transgressive segregation, with more extreme phenotypes eliciting a stronger response from the parental species (Rieseberg *et al.*, 1999; Pfennig, 2007; Abbot *et al.*, 2013; Rosenthal, 2013).

Considerable research has focused on the genetics of sterility and inviability in hybrids. The most consistent generalizations concern the genetics of postzygotic intrinsic barriers, in particular Haldane's rule (Haldane, 1922) and the large X effect (Coyne & Orr, 1989, 2004), which suggest that sex chromosomes play a key role in the establishment of post-zygotic barriers. By contrast, the genetics of behavioural sterility have been much less studied and further research is required to determine whether there are consistent patterns to the genetics of sexual isolation (Davies *et al.*, 1997; Etges, 2002). As early acting barriers, behaviours involved in mate location, recognition and courtship have the potential to strongly curtail gene flow, even in the absence of post-mating barriers (Seehausen *et al.*, 1997;

Arthur & Dyer, 2015), and likely play an important role in species isolation (Ritchie & Phillips, 1998; Coyne & Or, 2004).

We used a well-characterised field cricket system with XX/XO (female/male) sex determination to test whether the strength and direction of behavioural isolation varied across different potential stages of introgressive hybridization. The two species, *Teleogryllus commodus* and *T. oceanicus*, are a classic study system for acoustic sexual communication (Huber *et al.*, 1989). They overlap in an extensive area of what is believed to be secondary contact on the eastern coast of Australia and readily hybridize in the laboratory (Hill *et al.*, 1972; Otte & Alexander, 1983). However, genomic surveys have not found evidence of gene flow in the field (Moran *et al.* 2018). Long-range calling song has been suggested to be the primary barrier isolating these species (Hill *et al.*, 1972; Hoy & Paul, 1973; Pollack & Hoy, 1979; Hoy *et al.*, 1982; Hennig & Weber, 1997; Bailey *et al.*, 2017), but female discrimination against heterospecific song does not appear to be sufficiently precise to prevent heterospecific matings. Bailey & Macleod (2014) reported that when females of either species were given a choice between conspecific and heterospecific calling song, up to 25% of the time they chose the heterospecific song. When given no choice, Hill *et al.* (1972) found females of either species (from both allopatric and sympatric populations) exhibited high rates of phonotaxis to heterospecific song (*T. commodus* females 56 -58%; *T. oceanicus* females 40 – 55%). Taken together, the data suggest that multiple barriers contribute to maintaining the species' boundaries.

Hybrid females are sterile in both directions of crosses between *T. commodus* and *T. oceanicus* (Hogan & Fontana, 1973), providing a rare exception to Haldane's rule as females are the homogametic sex (Moran *et al.*, 2017). If both primary and secondary sexual trait development is disrupted in hybrid females this rare exception to Haldane's rule might also extend to behavioural sterility (Noor, 1997; Davies *et al.*, 1997). Since hybrid males are fertile, they potentially provide a gateway for inter-specific gene flow, so the ability of hybrids to elicit and enact successful matings is also of interest for predicting the extent and direction

of gene flow. Hybrid male song and preferences have been described in crosses between these species and there is some evidence that reciprocal hybrids and parental individuals would show assortative mating, possibly due to extensive sex-linkage of song and preference genes (Hoy & Paul, 1973).

Long-range mate attraction and close-range courtship behaviour in these species are multimodal processes, involving at least acoustic and chemical signals (Loher & Rence, 1978; Balakrishnan & Pollack, 1997; Bailey, 2011; Simmons *et al.* 2013). In both species, female phonotaxis towards male calling song is often followed by antennation and physical contact. Individuals gain information from physical contact with partners through cuticular hydrocarbons (CHCs), which are waxy molecules secreted on the cuticle and which play an important role in sex recognition and mate choice (Balakrishnan & Pollack, 1997; Bailey, 2011). In *T. oceanicus*, CHC profiles are heritable (Thomas and Simmons, 2008a), sexually dimorphic (Thomas & Simmons, 2008b) and are used by both males and females to discriminate against unattractive mates (Thomas & Simmons, 2009; 2010). Upon physical contact males produce courtship song which then elicits females to mount the male (Rebar *et al.* 2009). In *T. oceanicus*, courtship song may act as an indicator of mate quality (Zuk *et al.*, 2008; Simmons *et al.*, 2010; 2013). Once females have mounted the male, copulation lasts between 5-10 mins, during which time males make jerking movements which are necessary for threading the long spermatophore tube into the female genital tract (Alexander & Otte, 1967; Loher & Rence, 1978). After mating, females can remove the spermatophore, affecting sperm transfer success, as complete sperm transfer takes ca. 40 minutes in both species (Simmons *et al.*, 2003; Bussière *et al.*, 2006). Behavioural or mechanical isolation between species may occur at any stage during this sequence of courtship behaviours.

We performed two experiments, asking about the mechanisms of sexual isolation between these species and the genetic architecture of the three main sexual traits. In the first experiment, we focused on the outcome of close-range behavioural interactions among parental species and hybrids. We established how and at what stage of courtship species and hybrids are behaviourally isolated, and whether behavioural isolation is symmetrical.

Disruptions in courtship behaviour of hybrids would identify plausible mechanisms restricting interspecific gene flow. In the second experiment, we examined divergence between the two parental species and the reciprocal hybrids for three sexual traits: calling song, courtship song and CHCs. We evaluated genetic patterns of inheritance for each, and, using the F1 progeny produced, we tested for dominance and sex-linkage (or maternal effects). If F1 hybrid males and females exhibit intermediate sexual traits, it would suggest that these traits are based primarily on alleles acting additively in hybrids. However, intermediate phenotypes do not imply that individual loci show no dominance, if the species are fixed for dominant loci acting in opposite directions or if a trait is polygenic and only a few loci exhibit dominance or epistasis their effects will tend to cancel out in the hybrids (Falconer & Mackay, 1996). Comparisons between reciprocal hybrid males can be used to test if traits are sex-linked (cf. Hoy & Paul, 1973), as such males share the same complement of parental autosomes but differ in their X (Reinhold, 1998).

Methods

CRICKET ORIGINS AND REARING

The crickets used in this study originated from wild caught females from two allopatric Australian populations (*T. commodus*: near Moss Vale, NSW; *T. oceanicus*: near Townsville, QLD). We focused on allopatric populations to gain insight into barriers which could have contributed to RI upon secondary contact. Colonies were bred in the lab for three generations to reduce the potential for field-derived maternal effects. Stock crickets were housed in 16-L plastic boxes of ca. 80 individuals in a 25 °C temperature-controlled room on a photo-reversed 12:12 light:dark cycle. They were provided twice weekly with *ad libitum* Burgess Excel “Junior and Dwarf” rabbit food and cotton wool pads for drinking water and supplied with cardboard egg cartons for shelter.

BEHAVIOURAL TRIALS

No-choice mating experiments were conducted over two generations to investigate patterns of sexual isolation between parental species. To determine the strength and direction of premating barriers, the first generation comprised heterospecific and conspecific pairs. To test whether hybrids are sexually selected against, we then set up all combinations of backcrosses by pairing reciprocal hybrid males and females with individuals of each parental species. Close-range mating trials were conducted between 0900 and 1300 hours in a room dimly illuminated by red light and temperature controlled (23.0 - 25.5 °C). Virgin adult males and females 10-20 days post-adult eclosion were placed on opposite sides of a partition in a mating arena (22 x 27cm) with a sand base. Pairs were given a one minute acclimatization period to adjust to the new environment before the partition was removed and the trial started. Each trial lasted for ten minutes and if the pair made no physical contact within that period they were retested the next day. If no physical contact was observed during the retrial, the pair was removed from the analysis. The final dataset consisted of individuals that were only tested once.

Four courtship behaviours were scored as present or absent for each ten minute trial, thereby providing a proportional measure of courtship behaviour per cross type. The amount of behaviour exhibited during a close-range mating trial (count data) was also examined and the results were qualitatively the same, so we focus only on the proportion data here. The behaviours scored tended to occur sequentially as follows: courtship song ("song"), "mounting", "mating" and spermatophore transfer ("transfer"). "Mounting" was defined as when a female climbed on top of a male, whereas "mating" was assigned when males initiated the jerking movements associated with spermatophore transfer (Loher and Rence, 1978). We distinguished these two behaviours as they are likely to reflect different processes which could independently contribute to sexual isolation. If a spermatophore was transferred to a female during the ten minute trial, the trial was scored as successful.

QUANTIFYING REPRODUCTIVE BARRIERS

To quantify the relative strength of each mating behaviour to overall reproductive isolation, we calculated relative reproductive isolation (RRI) indices using a method devised by Coyne & Orr (1989), adapted by Ramsey *et al.* (2003) and similar to Veen *et al.* (2013) and Sobel & Chen (2014). This method compares the proportion of pairs which exhibited a response (song, mounting, mating, spermatophore transfer) in heterospecific crosses to the proportion exhibiting a response in conspecific crosses. The general form is:

$$RI = 1 - \frac{\text{Proportion of behaviour expressed among heterospecific pairs}}{\text{Proportion of behaviour expressed among conspecific pairs}} \quad (\text{Eqn. 1})$$

RI describes the relative strength of a barrier, where a value of 0 indicates no barrier and 1 represents a complete barrier. As earlier acting barriers have a greater potential to prevent successful matings, the absolute contribution (**AC**) represents the contribution of a barrier while accounting for the reduction due to earlier acting barriers (equations 1 – 4 in Ramsey *et al.* (2003)), such that:

$$AC_1 = RI_1 \quad (\text{Eqn. 2a})$$

$$AC_2 = RI_2 (1 - AC_1) \quad (\text{Eqn. 2b})$$

$$AC_3 = RI_3 [1 - (AC_1 + AC_2)] \quad (\text{Eqn. 2c})$$

More generally:

$$AC_n = RI_n (1 - \sum_{i=1}^{n-1} AC_i) \quad (\text{Eqn. 3})$$

Cross types were classified by two letter codes. The first letter indicates the maternal species and the second the paternal species (CC = pure *T. commodus*; OO = pure *T. oceanicus*, OC = hybrid offspring from a female *T. oceanicus* and male *T. commodus* cross, while CO represents offspring from the reciprocal cross). The experimental design was similar across both generations. However, in the first generation male and female crickets had been isolated for ten days prior to courtship trials, while in the second generation due to the difficulty of isolating larger numbers of crickets, males and females were instead maintained in sex segregated boxes of ca. 50 individuals prior to the trials. Direct comparisons across the two generations should therefore be made with caution.

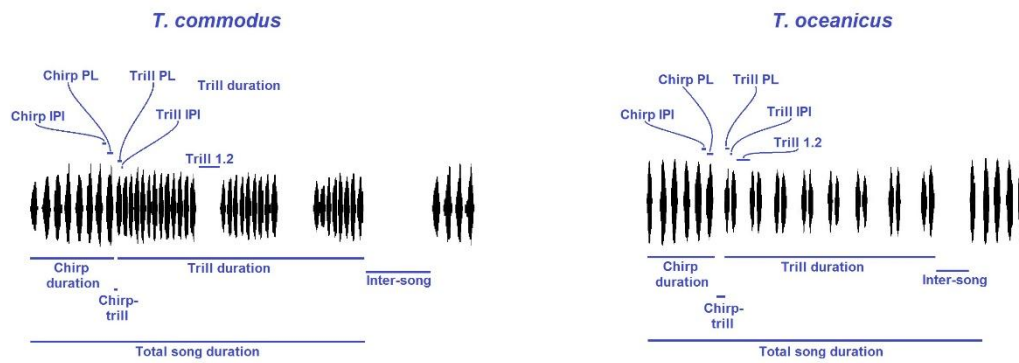
MEASURING TRAIT DIVERGENCE

Song Recording and Analysis

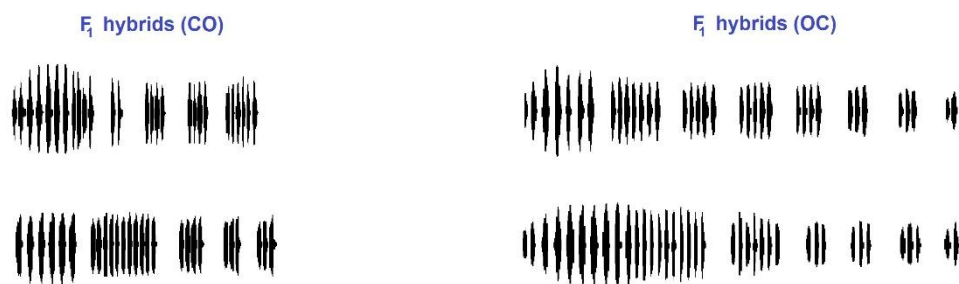
Parental species and reciprocal hybrid individuals were phenotyped for calling song, courtship song and CHCs (Table S1). The CHC profiles and calling song recordings were based on individuals that were not used in the close-range mating trials, because close physical contact could contaminate the target males' CHC profiles. In contrast, courtship song was recorded from males used in the close-range mating trials as its production is dependent upon close physical contact. Both calling and courtship songs were recorded using a Sennheiser ME 66 microphone under dim red light, in a temperature controlled room (22 - 26 °C). The males used were ca. 10-20 days post-adult eclosion. Calling songs were recorded from males isolated in 118-mL plastic containers. Approximately one minute of song was recorded per individual, and five song phrases analysed to obtain an individual's average. All songs were analysed using Sony Sound Forge (7.0). Both spectral and temporal call elements were measured for both song types (calling song parameters $n = 14$,

courtship song parameters $n = 12$). Measured song parameters are illustrated in Figures 1a and b and detailed in Tables S4 & S6. Two song parameters “Trill.1.2.interval” and “Intersong.Interval” were dropped from the courtship song analysis due to their high variability.

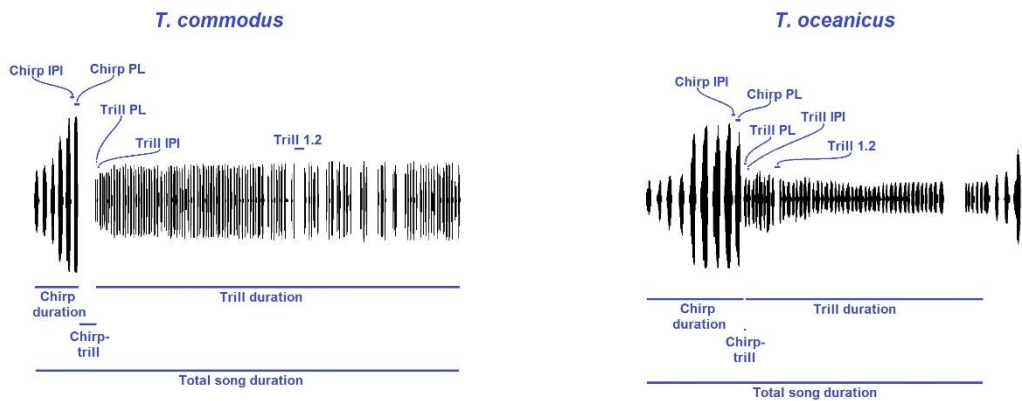
A. Parental calling songs



B. Hybrid calling songs



C. Parental courtship songs



D. Hybrid courtship songs

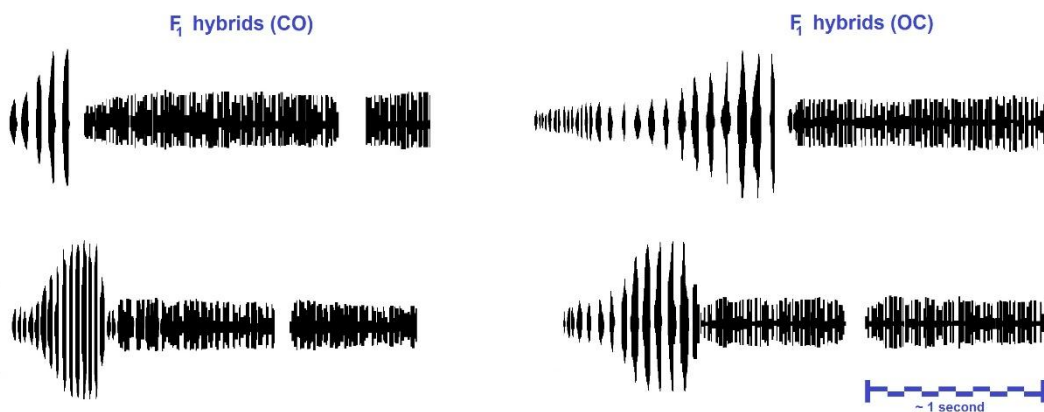


Figure 1. Diagrams of male song, re-drawn from original recordings. (A) Male calling song of both parental species with key song parameters labelled (adapted from Bailey & Macleod, 2014). (B) Representative examples of calling songs from reciprocal hybrid males. Two examples are given for each cross direction (CO = F₁ hybrid offspring from a female *T. commodus* and male *T. oceanicus* cross; CO represents the reverse cross). (C) Male courtship song of both parental species with key song parameters labelled. (D) Examples of courtship songs from reciprocal hybrid males, with two examples for each cross direction. The *T. commodus* and hybrid courtship songs were truncated to fit in the diagram.

Cuticular Hydrocarbon Assays

CHC extraction and analysis followed previously-published protocols (Pascoal *et al.* 2016). CHCs were extracted from virgins of each sex, for each parental species and their reciprocal hybrids ca. 10-20 days post-adult eclosion. Individuals were isolated in small (118 mL) plastic containers for ca. 10 days, provisioned with Burgess Excel “Junior and Dwarf” rabbit food and water, before being anesthetized by chilling and then placed in 4mL glass extract vials (QMX Laboratories) and stored at -20 °C. Prior to CHC extractions, samples were thawed at room temperature. An aliquot of 4 ml of HPLC-grade hexane (Fisher Scientific) was pipetted into each vial and left for 5 minutes before removing the cricket with clean forceps. A 100 µl sample of this liquid extract was pipetted into 0.3mL fixed insert vials (Chromacol LTD, Item # 11573680) and left to evaporate under a fume hood overnight. Samples were subsequently reconstituted using 100µl of hexane containing 10 ng µL⁻¹ dodecane as an internal standard.

Gas chromatography was performed using an Agilent 7890A GC coupled with an Agilent 5975C mass spectrometer, and an HP-5ms column (30 m x 0.25 mm x 0.25 µm). A 2 µl aliquot of extract was injected into a multimode inlet operating in pulsed splitless mode, at

a temperature of 250 °C. Hydrogen was used as the carrier gas at a column flow rate of 1ml/min. The oven temperature profile was as follows: 70 °C for 1 min, ramping at 20 °C/min to 250 °C, then 4 °C/min to 320 °C with a 5 minute hold. The MS transfer line was set at 280 °C. Mass spectra were obtained at a scan range of 40-500 m/z. Data analysis was performed using Agilent MSD Chemstation E.02.02.1431.

Statistical Analysis of Traits

Statistical analyses were performed in R (Version 3.1.3). Behavioural data, which were recorded as proportions of pairs exhibiting particular courtship behaviours, were analysed using generalized linear models (GLM) with binomial distributions (with the exception of song traits from both generations, and all traits in the second generation, which were analysed using quasibinomial distributions due to overdispersion of the data). We fitted the following as fixed effects: the focal individual's species identity (i.e. identity of the male species for "song" or female species for the other behaviours) and cross type (e.g. "conspecific" or "heterospecific" in the first generation). The weight of individuals was included as a covariate. For the second generation, "mating type", referring to the mating pair's identity (e.g. OO x CC), was fitted as a fixed effect. The decision to include or remove a variable from the model was made based on comparison of model fit using likelihood ratio tests. To determine if groups of interest differed, Tukey pairwise comparisons were implemented which account for multiple testing.

Some prior work has analysed the contributions of specific song variables to female preferences (e.g. Brooks et al. 2005; Bailey et al. 2017), but this has focused on male calling song and examined a smaller number of traits than we measured in the present study. The inability to identify the contributions of key underlying trait components to the expression of relevant behaviours in receivers is a particularly acute problem for CHC profiles (Blows & Allan, 1998), and data on perception and discrimination of individual CHC peaks is lacking in *Teleogryllus* species. We therefore used PCA implemented in the R package *FactoMiner* (Lê

et al., 2008) to reduce the dimensionality of our data and facilitate subsequent analyses of genetic inheritance patterns (e.g. Henry et al. 2002). For song data, temperature-corrected residuals were used in PCAs to control for temperature differences between song recordings. For CHCs, data were standardized prior to analysis by dividing the abundance of each peak by the internal standard (10 ng μL^{-1} dodecane) and normalized using a \log_{10} transformation. A total of 14 CHC peaks were used in the analysis, and we only included those peaks which were detectable (non-zero abundance values after integration) in at least 60% of the individuals within each cross type, to avoid analysing peaks for which data might be predominantly absent in any particular cross type. Table S10 and Figure S3 describe the number of CHC peaks retained and the pattern of variation when different filtering thresholds, based on the amount of zero values allowed, were applied.

Multivariate analysis of variance (MANOVA) and post-hoc pairwise comparisons (glht function) was performed on PC scores to test the degree of divergence between the main groups of interest (species, sex, or species*sex) using the R *multcomp* package (Hothorn et al., 2008). MANOVA provides a useful overall indication of variation among groups of interest when multiple traits are under consideration, but given that the 'traits' entered into this analysis were PCs, we separately verified that MANOVAs performed on raw calling song, courtship song, and CHC data yielded consistent qualitative patterns among cross types, and in the case of CHCs, sexes (Table S11).

INHERITANCE PATTERNS OF BEHAVIOURAL TRAITS

X-linkage

The influence of X chromosomes on interspecific differences for male sexual traits (the heterogametic sex) was estimated using the mean trait differences between F1 reciprocal hybrid crosses divided by the difference between the two parental lines (Reinhold, 1998; Oh et al., 2012):

$$I_x = \frac{C_{AxB} - C_{BxA}}{C_A - C_B} \quad (\text{Eqn. 4})$$

where C_A and C_B are the trait means for the males of parental lines **A** and **B**, respectively, and C_{AxB} and C_{BxA} for reciprocal hybrids. The normalized index of I_x enables comparisons of the degree of X-linkage among different traits (Reinhold, 1998). An I_x of 0 indicates no X-linkage of the trait in question, while an I_x of 0.5 reflects half of the phenotypic difference between the parental lines being caused by X-linked genes (or maternal effects), and a value of 1 is consistent with all of the differences being due to X-linked genes (or maternal effects). We only inferred support for sex-linkage if reciprocal hybrid types were significantly different and if the difference was in the direction of their maternal species. In addition to examining the multivariate trait means, we also tested for sex-linkage of the univariate trait trill interval ("Trill 1.2" cf. Figure 1) as this component was previously suggested to be sex-linked (Bentley & Hoy, 1972).

Transgressive Segregation

F1 hybrid phenotypes are expected to be intermediate to those of the parental species if most gene interactions are additive. The extent of departure from intermediacy due to directional dominance (heterosis) can be estimated by the difference between the mean F₁ trait value M_{F1} and the mid-parent mean value $M_{\bar{P}}$ (Falconer & Mackay, 1996; p 255). The estimate of heterosis can be positive or negative. The term heterosis is often used in context of increased hybrid fitness, but here we use it solely to refer to deviations from the midparent value in hybrid behaviours. The mid-parent value is given by:

$$M_{\bar{P}} = \frac{1}{2} (M_{P1} + M_{P2}) \quad (\text{Eqn. 5a})$$

and the amount of heterosis is given by:

$$H_{F1} = (M_{F1} - M_{\bar{P}}) \quad (\text{Eqn. 5b})$$

Following Rieseberg *et al.* (1999), traits were defined as transgressive if the character means lay outside the means of both parental species in a negative or positive direction. Traits were classified as dominant if they exhibited a significant difference in Tukey pairwise contrasts from one of the parental species, but were indistinguishable from the other. In all other cases, traits were classified as intermediate. Sample sizes for the sexual trait measurement and inheritance experiment are given in Table 1.

Table 1. Cross types and sample sizes for sexual trait measurement and inheritance experiment. Song is a male-limited trait, while CHCs were extracted from both males and females. Designation of cross types follows the in-text terminology.

Trait	CC		CO		OC		OO	
	♀	♂	♀	♂	♀	♂	♀	♂
Calling Song	-	19	-	12	-	13	-	19
Courtship Song	-	16	-	8	-	8	-	13
CHCs	10	10	12	18	14	14	16	12

Results

INTERSPECIFIC SEXUAL ISOLATION IS SYMMETRICAL

Heterospecific pairs had a lower tendency to exhibit courtship behaviours (Figure 2, Tables 2, 3 and S2). A large proportion of males started singing when paired with a conspecific female (OO: 0.56, CC: 0.72), but only a small proportion of pairs successfully transferred spermatophores (OO: 0.12, CC: 0.19) (Figure 2). The strongest difference between heterospecific and conspecific pairs was for courtship song. In particular, *T. oceanicus* males paired with heterospecific females exhibited a marked reduction in the tendency to produce courtship song (Figure 2, Table S2). Females of both species exhibited a similar tendency to

mount or mate with heterospecific males at a lower rate than with conspecific partners.

Overall, non-significant interactions between cross type and male or female identity in models examining effects of cross type on courtship behaviours indicated that responses of *T. commodus* and *T. oceanicus* paired with conspecific versus heterospecific partners were indistinguishable (Table 3). Behavioural isolation was virtually complete between the parental species, with a total **RI** for OO x CC crosses = 0.993, and CC x OO = 1.

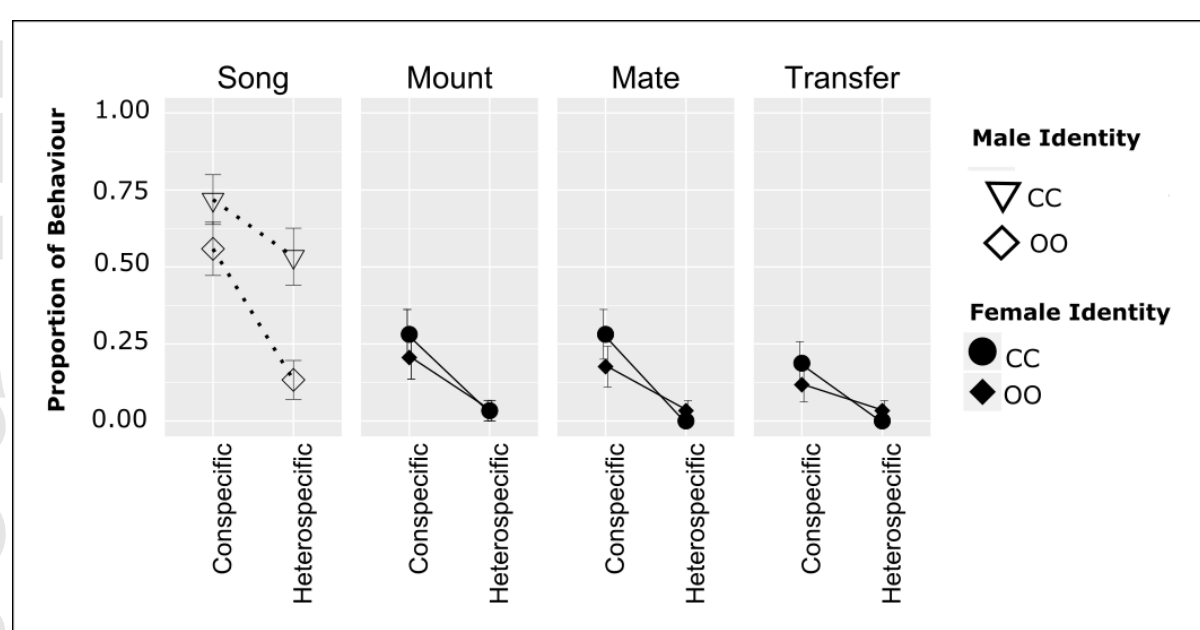


Figure 2. Close-range courtship, contrasting the proportion of trials in which behaviours were observed in conspecific versus heterospecific pairings. Symbols indicate focal individual species identity (CC = *T. commodus*, OO = *T. oceanicus*). The broken line in the first panel indicates that “Song” is a male sex-limited trait. Error bars represent standard error.

Table 2. Components of reproductive isolation, ***RI*** (Eqn. 1) and their absolute contribution, ***AC*** (Eqn. 3) for each courtship behaviour. ***AC*** measures the contribution of each barrier while accounting for reductions due to earlier acting barriers. The proportion of dyads exhibiting each behaviour are compared between conspecific and heterospecific pairings. Song is male-limited, so the conspecific reference is the paternal species. The other behaviours are female controlled, so the reference is the maternal species.

Barrier	Components of reproductive isolation (<i>RI</i>)		Absolute contribution to reproductive isolation (<i>AC</i>)	
	OO x CC (♀ x ♂)	CC x OO (♀ x ♂)	OO x CC (♀ x ♂)	CC x OO (♀ x ♂)
Song	0.224	0.761	0.224	0.761
Mount	0.838	0.867	0.650	0.207
Mate	0.811	1	0.102	0.032
Transfer	0.716	1	0.017	0
Total			0.993	1

Table 3. Results of generalized linear models examining the frequency of four courtship behaviours among conspecific and heterospecific pairs (“cross type”). After model selection, the final model included significant or nearly significant effects which are indicated below. All models were conditioned on song to account for the sequential nature of the behaviours. F-tests were used for song as quasibinomial models were fitted, whereas chi-square tests were fitted for the other binomial traits. Tukey mean contrasts (glht) were used to test for “cross type” differences and are provided in the Supplementary section (Table S2).

courtship behaviour	model selection	df	F	p
song				
	cross type	1,124	11.503	<0.001
	male identity	1,123	10.458	0.002
mount				
	cross type	1,123	76.618	0.012
	male weight	1,122	71.875	0.029
mate				
	cross type	1,123	70.032	0.003
	male weight	1,122	64.745	0.021
transfer				
	cross type	1,123	60.41	0.031
	male weight	1,122	54.371	0.014

HYBRIDS ARE NOT BEHAVIOURALLY STERILE

Backcrosses with hybrid males and females all exhibited relatively high rates of courtship behaviour (Figure 3). A large proportion of males produced courtship song, irrespective of whether they were a hybrid male (ranging from 0.50 - 0.80) or a male of either parental species (0.65 - 0.80). Hybrid females also mounted (0.45 - 0.70) and mated (0.20 - 0.35) at a relatively high rate. None of the factors, including mating type, predicted the frequency of courtship behaviours (Table S3), and parental species (CC and OO) and reciprocal hybrid pairs (OC and CO) did not differ. This may partly reflect a higher variance in courtship behaviours among hybrid individuals. The proportions of pairs exhibiting courtship behaviours were higher than those observed among the conspecific crosses in the first generation of crosses (Figure 3). This may reflect an increased propensity for hybrids to mate (i.e. hybrid vigour), but experimental differences in the rearing of the crickets between the two generations preclude a formal comparison. Taken together, the results indicate that hybrids of both sexes are capable of eliciting and enacting courtship behaviour, despite the fact that hybrid females are sterile (Moran *et al.*, 2017).

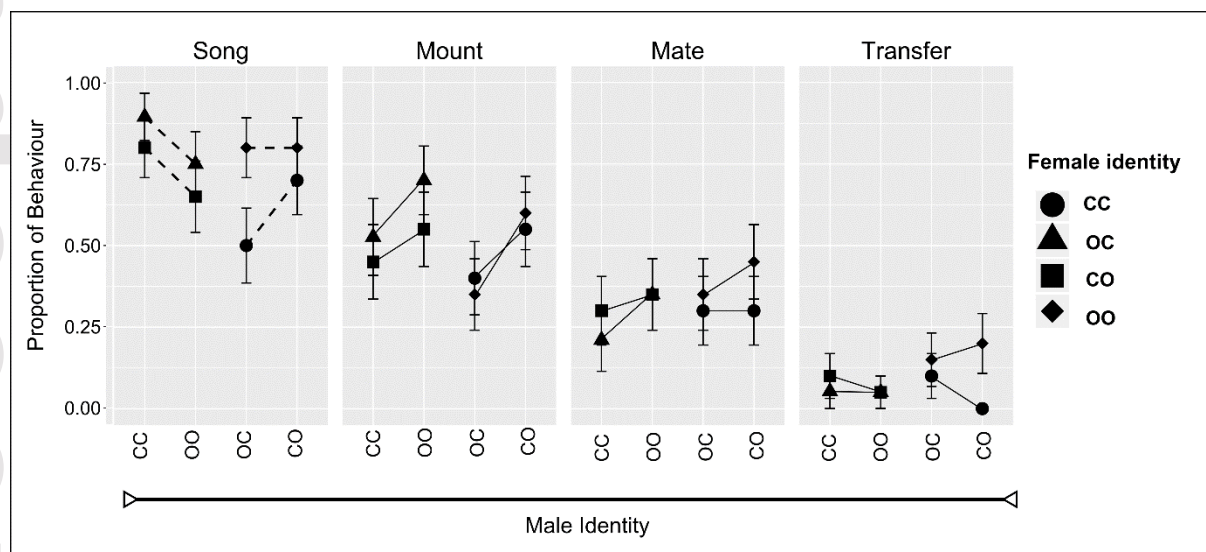


Figure 3. Close-range courtship trial results: proportion of trials in which courtship behaviours were observed amongst backcross pairs. Symbols indicate the female species identity and the X-axis indicates the male's identity (CC = *T. commodus*; OO = *T. oceanicus*, OC = hybrid offspring from female *T. oceanicus* and male *T. commodus* crosses, CO = reverse cross). "Song" is distinguished by dotted lines as it is a male sex limited trait, whereas the other behaviours are principally under female control. Error bars represent standard error.

PATTERNS OF SEXUAL TRAIT DIVERGENCE AND INHERITANCE

Calling song

Figures 4a and 5a and Table 4 describe variation in the long-range calling song produced by males of the parental *Teleogryllus* species and their hybrids. Multivariate analysis on the scores from the first four principal components (eigenvalues > 1), which cumulatively account for 78.22% of the total variation, revealed significant calling song differences among the four cross types (MANOVA: Wilks' $\lambda = 0.043$, $F_{3,59} = 28.265$, $p < 0.0001$). The first principal component (PC1) contrasted trill and frequency components, the second component (PC2) was dominated by song length components while the third component (PC3) contrasted the chirp and trill duration (Table S4). PC1, which accounted for 38.47% of the variation, clearly separated the parental species, whereas PC2 (19.84% of the variation) predominantly differentiated the reciprocal hybrids (Figure 4a). All *post-hoc* Tukey comparisons for calling song are provided in Table S5. Hybrid males from both cross directions were intermediate between the parental species for PC1, and distinguishable from the parental species (Figure 4a). For PC2, hybrids with a *T. oceanicus* X chromosome (OC) exhibited more extreme trait values compared to either of the parental species and to the hybrids from the other cross direction (Table 4). We found no support for sex-linkage of PC1 song components ($I_x = -0.032$) using the I_x index (Reinhold, 1998). For PC2, there was a large difference between the reciprocal hybrids (Figure 4). CO hybrids were most similar to

their maternal species, however OC hybrids were most different from their maternal species ($I_X = -1.54$). For PC3, even though the I_X index was relatively high (1.69), there the reciprocal hybrid crosses did not differ from the parental species, suggesting the associated song components may not be sex-linked. Instead, the pattern of inheritance for calling song PC3 components was more consistent with heterosis (H_{F1} : OC = -1.228; CO = -0.747). We had an *a priori* expectation of sex-linkage for the univariate song trait, “Trill 1.2” (Bentley & Hoy, 1972), but in a single-trait analysis we found no evidence for such sex-linkage ($I_X = -0.084$). Instead, “Trill 1.2” was almost perfectly intermediate between parental species’ values (H_{F1} : OC = -0.006; CO = -0.063) (Figure S1).

Courtship song

Figures 4b and 5b and Table 4 describe male courtship song variation in *Teleogryllus* parental and hybrid individuals. The first four principal components (eigenvalues > 1) describing courtship song traits cumulatively accounted for 76.23% of the total variation. Courtship song differed significantly among the four different cross types (MANOVA: using “cross type” as a fixed effect, Wilks’ $\lambda = 0.120$, $F_{3,38} = 9.5$, $p < 0.0001$). PC1 was dominated by song length elements and pulse durations, while PC2 contrasted frequency and number of trills against chirp elements (Table S6). All *post-hoc* Tukey comparisons for courtship song are provided in Table S5. PC1, which accounted for 31.96% of the total variance, varied between parental species (Figure 4b), but most of the variation in PC1 was due to differences between hybrid types (Figure 4b). In particular, hybrid males with a *T. commodus* X chromosome (CO) exhibited trait values more extreme than either of the parental species (H_{F1} : 2.338), whereas OC males were intermediate (H_{F1} : -0.251) (Figure 4b). The greatest difference was between CO males and the paternal species *T. oceanicus*, and we found strong support for sex-linkage ($I_X = 1.05$) for song elements associated with PC1 (Table 4). For PC2, the parental species were distinguishable and the reciprocal hybrids exhibited an intermediate pattern between that of both parental species (H_{F1} : OC = -0.249, CO = 0.038)

(Figure 4b). Reciprocal hybrid crosses did not vary and the parental species differences showed no evidence of X-linkage ($I_x = -0.144$) (Table 4). For PC3, the greatest difference was between the reciprocal hybrid cross types, which were almost outside the range of both parental species. Although the I_x index was relatively high, both hybrid cross types were most dissimilar to their maternal species which is inconsistent with sex-linkage (Table 3).

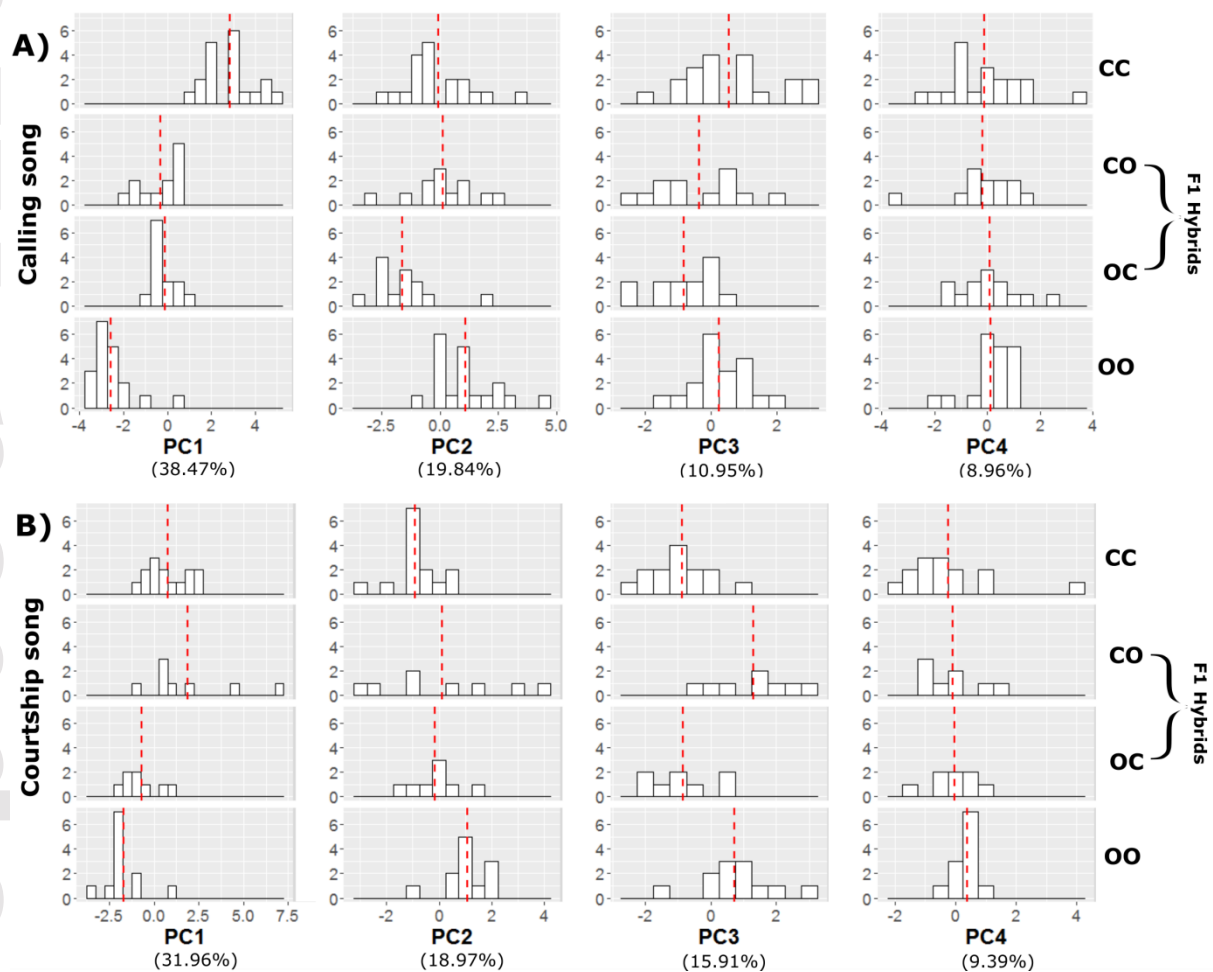


Figure 4. Histograms illustrating variation in (A) calling song and (B) courtship song between parental *Teleogryllus* species and their hybrids. For both song types, principal component scores are given for the parental species (CC – *T. commodus*; OO – *T. oceanicus*) and their reciprocal hybrids (CO – male hybrids with a *T. commodus* X chromosome; OC – male hybrids with a *T. oceanicus* X chromosome). Red dotted lines indicate group means.

Cuticular hydrocarbons

Figures 5c and 6 and Table 4 describe variation in the CHC profiles of parents and hybrids of both sexes. Table S7 provides significant loadings for each CHC peak, peak identifications, and retention times, and figure S2 provides example chromatographs for each parental and reciprocal hybrid cross type. Multivariate analysis on the scores from the first 5 principal components (eigenvalues > 1), which accounted for 66.94% of the total variation in CHC data, revealed significant sex differences (MANOVA: Wilks' λ = 0.667, $F_{1,98}$ = 9.417, p < 0.0001), variation among cross types (Wilks' λ = 0.621, $F_{3,98}$ = 3.268, p < 0.0001) and a cross type*sex interaction indicating that the pattern of sex bias was uneven between different hybrid crosses (Wilks' λ = 0.518, $F_{3,98}$ = 4.657, p < 0.0001). All Tukey *post-hoc* comparisons for CHCs are provided in Table S8. The extent of sexual dimorphism was more prominent in *T. oceanicus*, with both sexes distinguishable on PC1 and PC2, while in *T. commodus* both sexes were only distinguishable on PC3 (Figure 6, Table S8). When comparing the sexes of both parental species, only males were distinguishable, and only on PC4 (Table S8). The greater magnitude of differences between the sexes rather than the parental species may reflect an underestimation of group differences due to the removal of more divergent CHC peaks with a large amount of zero values ($\geq 40\%$ per cross type). When the filtering threshold for the allowable amount of zero values per cross type was relaxed, a greater number of peaks were retained (Table S10) and PCA revealed that most of the variation distinguished the parental species (Figure S3).

Hybrids did not exhibit an intermediate pattern for PC1, as expected for a polygenic additive trait (Figure 6). Instead, hybrid CHC profiles exhibited dominance, sharing a strong similarity with the parental species *T. commodus*: male hybrids differed from *T. oceanicus* individuals but were indistinguishable from *T. commodus* individuals (Figure 6). PC2 and PC3 showed large sex differences, even within the same hybrid crosses (Figure 6). However, we found no support for sex-linkage of CHC components associated with any of the PCs as reciprocal hybrid males did not vary. Hybrids exhibited extremely variable CHC

profiles, which appears to have resulted in little distinguishable variation among hybrids and the parental species for individuals of the same sex (Table S8).

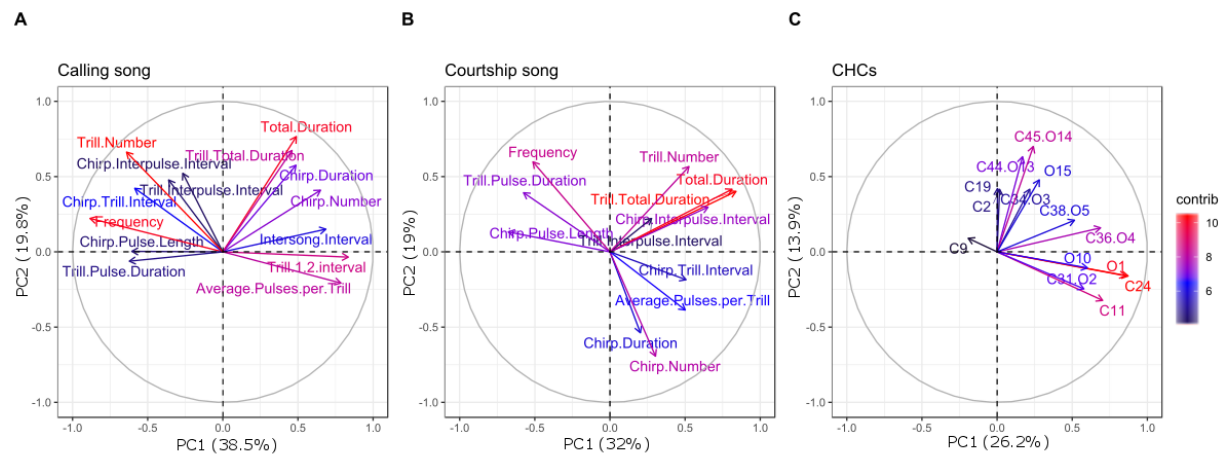


Figure 5. Variable factor maps illustrating the strength and direction of loadings for the constituent variables loading on PCs 1 and 2 for (A) calling song, (B) courtship song, and (C) CHCs. Vectors show the relative importance of contributing variables, with longer arrows and redder colours indicating the most strongly loading variables. For example, nearly all song traits load heavily on PC1, whereas CHC components are less consistently heavily loaded but are almost all positive on PC1, suggesting PC1 provides information about overall amount of CHCs expressed. Variable labels correspond to Figures 1 and S2.

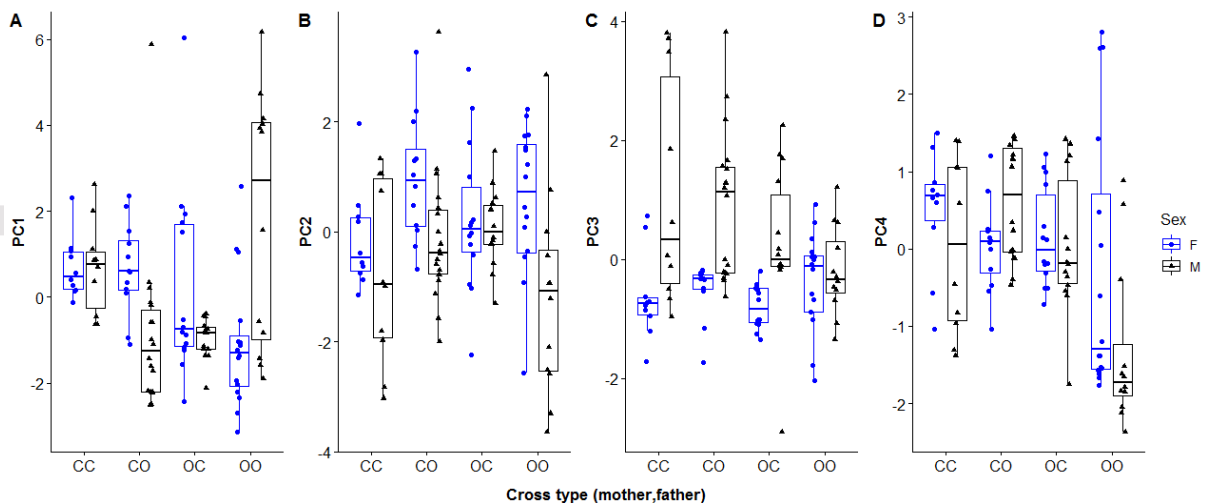


Figure 6. Variation in CHC profiles among *Teleogryllus* parental species and their hybrids based on the first four PCs (panels A-D show PCs 1-4, respectively). PC scores are plotted on the y-axes, colours and shapes correspond to females (blue circles) and males (black triangles), and cross types are indicated along the x-axes. Group medians are indicated by horizontal lines, boxes show first and third quartile, and bars represent 95% confidence intervals.

Table 4. Summary data for phenotypic variation in three sexual traits among parental *Teleogryllus* species and their hybrids, including estimates of heterosis (H_{F1}) and sex-linkage (I_X). For each trait, means \pm SE are given for principal components in supplementary Table S9.

		PC1	PC2	PC3	PC4	PC5
Calling song	Eigenvalue	5.385	2.778	1.533	1.255	
	Variance (%)	38.466	19.84	10.952	8.964	
	H_{F1} : CO	-0.431	-0.367	-0.747	-0.188	
	H_{F1} : OC	-0.256	-2.148	-1.228	0.078	
	I_X	-0.032	-1.543	1.688	1.039	
Courtship song	Eigenvalue	3.836	2.276	1.909	1.127	
	Variance (%)	31.964	18.968	15.909	9.388	
	H_{F1} : CO	2.338	0.038	1.3645	-0.16	
	H_{F1} : OC	-0.251	-0.249	-0.7815	-0.098	
	I_X	1.054	-0.144	-1.357	0.099	
CHCs	Eigenvalue	3.675	1.946	1.436	1.269	1.044
	Variance (%)	26.25	13.903	10.257	9.068	7.460
	H_{F1} : CO_F	0.865	0.754	-0.005	-0.080	0.231
	H_{F1} : CO_M	-2.133	0.847	0.440	1.245	0.709
	H_{F1} : OC_F	0.409	0.026	-0.290	0.032	0.356
	H_{F1} : OC_M	-2.231	1.027	-0.177	0.688	0.200
	I_X	-0.084	-0.415	0.467	0.408	-0.738

Discussion

How consistent is symmetrical species discrimination in different sexual signal modalities, and can it inform us about the evolution of species differences leading to behavioural isolation? Here we found that in sister species of Australian field crickets, *Teleogryllus commodus* and *T. oceanicus*, both hybrid males and females elicited and enacted courtship behaviours at a relatively high rate (Figure 3). Hybrid females are sterile (Moran *et al.*, 2017), but our findings suggest that gene flow could occur via backcrosses with male hybrids, so any lack of gene flow observed in the field is unlikely to reflect behavioural sterility of F_1 hybrids. The parental species showed symmetrical behavioural isolation involving both long and close-range male sexual signals, against which heterospecific females of each species exhibited a similar tendency to discriminate.

The timing of divergence and secondary contact (or parapatric speciation) among Australian *Teleogryllus* species is unknown, and their distribution could reflect recent range expansions promoted by human disturbance (Cairns et al., 2010) or a more ancient Holocene expansion from glacial refugia (Moritz et al., 2009). Our use of allopatric populations in this study provides insight into behavioural barriers which could have contributed to reproductive isolation in either case, although the lack of comparison with sympatric populations limits our ability to fully assess the role of behavioural barriers in curtailing gene flow between the species. Behavioural barriers might be predicted to be even stronger in sympatry due to reinforcement, given that hybridization between *T. commodus* and *T. oceanicus* is costly (Moran et al., 2017), but a study by Hill et al. (1972) comparing allopatric and sympatric populations found no evidence for character displacement in either male calling song or female preferences. The strong behavioural isolation we detected among the parental species, combined with the apparent absence of both character displacement (Hill et al., 1972) and hybrids in the field (Moran et al., 2018) suggests that premating isolation may be the primary barrier maintaining the species boundary.

Are there consistent patterns to the genetics of sexual isolation? Behavioural barriers may share certain genetic features with classic intrinsic barriers (i.e. physiological sterility and inviability) such as reduced (or disrupted) behaviour in the heterogametic sex (Haldane's rule), asymmetric isolation (Darwin's corollary to Haldane's rule: Turelli & Moyle, 2007) or disproportionate X effects (large X effect). Only a few examples of Haldane's rule extending to sexual isolation have been documented (Davies et al., 1997, Noor, 1997). In *Teleogryllus* crickets the genetic factors which disrupt hybrid female fertility do not appear to negatively affect secondary sexual trait development or expression, as hybrid males and females exhibited the same propensity to mate with partners of either parental species (Figure 3). Therefore this rare exception to Haldane's rule does not appear to extend to behavioural sterility in *Teleogryllus*.

Behavioural isolation has been found to be asymmetric in a number of taxa, and several mutually non-exclusive theories have been proposed to explain these asymmetries (reviewed in Arnold et al., 1996; Coyne & Orr, 2004; Svensson *et al.*, 2016). Asymmetries in prezygotic barriers appear to occur at a lower rate than in postzygotic barriers (Lowry *et al.* 2008; Veen et al. 2013). Prezygotic barriers may be expected to exhibit less consistent patterns due to the complexity of traits involved, whose development, expression and fitness is often dependent on the environment (Grant & Grant, 1993; Pfennig, 2007), in contrast to intrinsic genetic incompatibilities which may be more functionally constrained (Turelli & Moyle, 2007). In Australian *Teleogryllus*, we found behavioural isolation to be symmetrical. This may reflect the allopatric origin of the populations we studied. Further comparative work is needed to determine whether asymmetries in prezygotic barriers are more common or predictable in certain groups than others (e.g. in sympatry vs. allopatry, Yukilevich, 2012).

Our results highlight an important distinction between Australian *Teleogryllus* species in the strength of male vs. female discrimination. Differences in discrimination by males and females may indicate divergent selection pressures between the sexes (Svensson et al., 2007), and females are generally expected to be the more discriminating sex due to predicted higher fitness costs for mating with incompatible mates (Andersson, 1994). However, sex roles during mate choice can be highly dynamic due to differences in the relative costs of mating (reviewed in Gwynne, 1991; Bonduriansky, 2001). The system of mutual mate choice in these species seems particularly interesting (Thomas & Simmons, 2010), as in many species research primarily (or exclusively) considers female choice and/or male-male competition. Male *T. oceanicus* discriminated against heterospecific females upon contact, indicated by the females' failure to elicit courtship song, while male *T. commodus* did not exhibit as strong a reduction in singing behaviour when paired with a heterospecific female. Instead, the main barrier preventing *T. commodus* males mating with heterospecific females is due to females declining to mount. Thus, while behavioural isolation may be approximately equal, and strong, in the two species, the behavioural mechanisms by which this isolation is exerted appear to differ. This mirrors findings that

female *T. commodus* and *T. oceanicus* process and discriminate between species-specific male calling songs using divergent neurophysiological pattern-recognition mechanisms (Bailey *et al.*, 2017).

Genes underlying sexually selected traits are predicted to accumulate on sex chromosomes (Charlesworth *et al.*, 1987) but empirical results supporting this have been mixed (Reinhold, 1998; Qvarnström & Bailey, 2009). Previous studies of these species have implicated sex-linked song and preference divergence (Hoy *et al.* 1973). Our analyses of male sexual signals in *Teleogryllus* crosses revealed patterns of inheritance consistent with sex-linkage for three out of thirteen principal components studied. For courtship song PC1 and CHC PC2 components, hybrid crosses diverged in the direction of their maternal species, as expected for X-linked traits. However, for components associated with calling song PC2 and courtship song PC3, OC individuals were most dissimilar to their maternal species *T. oceanicus*. This makes it difficult to distinguish the effects of X-linkage from non-symmetric heterosis (Reinhold, 1998), and the differences observed amongst reciprocal hybrids could be due to either sex-linkage and/or maternal effects. To properly distinguish these confounding effects, further work would be required to examine trait segregation over additional backcross generations (Butlin & Ritchie, 1989).

Calling song and courtship song components were predominantly intermediate among hybrids. However, hybrid phenotypes were outside the range of the parental species for some song components, indicating transgressive segregation (Figure 4: PC2 & PC3 for calling song and PC1 for courtship song). In contrast, CHCs primarily exhibited dominance and transgressive segregation. This apparent distinction between the two signal modalities may reflect differences in genetic architectures and the histories of trait selection. Previous studies have suggested many genes of small effect predominate acoustic communication systems while chemical communication is more likely to be influenced by major effect loci (Ritchie & Phillips, 1998; Shaw *et al.*, 2011; Ellison *et al.*, 2011). Transgressive segregation may be more frequent when single genes can have large effects, resulting in novel mating signals and preferences, with important evolutionary consequences such as adaptive

hybridization and even hybrid speciation (Rieseberg *et al.*, 1999; Seehausen, 2004; Jiggins *et al.*, 2008; Abbott *et al.*, 2013; Rosenthal, 2013). A review by Rieseberg *et al.*, (1999) examining trait segregation among plant and animal hybrids revealed that transgressive segregation occurred in 78% of animals studied (45 of 58 cases) and 97% for plants (110 of 113 studies). Extreme phenotypes in wild outbred populations have been found in roughly a quarter of animal taxa and a third of plant taxa (Arnold *et al.*, 1999; Abbott *et al.*, 2013). Such transgressive segregation appears to be caused by complementary gene action and may be more pronounced in traits with a few genes of large effect (Rieseberg *et al.* 2003).

The history of selection acting on the trait is likely to influence the occurrence of transgressive segregation. Countervailing natural selection on CHC profiles is likely to differ considerably from that on male song, due to the involvement of CHCs in other functions such as desiccation resistance. In addition, CHCs are known to be plastic and dependent on social context (Pascoal *et al.* 2016), meaning that the genetic architecture and patterns of behavioural isolation mediated by CHCs are likely to be environmentally dependent. Traits with a history of directional selection may be less likely to exhibit transgressive segregation than those with a history of stabilizing selection, as directional selection will lead to the fixation of alleles whose effects are in opposing directions, which in hybrids will cancel out producing intermediate trait patterns (Rieseberg *et al.*, 1999). The fact that transgressive segregation in CHC profiles appears to increase sexual dimorphism (Figure 6) is unexpected. It may suggest an interaction between sexual differentiation and transgressive segregation, and could provide important material for sex-specific selection, including reinforcement.

Conclusions

Close-range behavioural barriers play a key role in species isolation. We found that sister species of Australian *Teleogryllus* exhibited symmetrically reduced rates of courtship behaviour, indicating an important role for behavioural isolation in maintaining species

boundaries. Behavioural isolation was almost complete, yet hybrid males and females appeared to enact and elicit relatively high rates of courtship even though the latter are sterile. Therefore, hybrid behavioural sterility cannot explain the lack of gene flow in the field and the exception to Haldane's rule that manifests in this species pair does not extend to sexual isolation. The apparent absence of hybrids in sympatric populations (Moran *et al.*, 2018), suggests pre-mating barriers between parental species are sufficiently strong to prevent hybridization. Even though components of signal traits exhibited greater similarity to one or the other of the parental species, in particular for CHC profiles, we found no corresponding asymmetry in mate choice in behavioural trials. Inheritance patterns for song traits were primarily intermediate, whereas CHC profiles exhibited evidence of dominance and sex-specific transgressive segregation. It is difficult to determine whether these behavioural barriers may have preceded the species' secondary contact, but differences between allopatric populations support a role for intraspecific processes, such as sexual selection, in driving the divergence of these sexual traits. Overall, our results support the view that close-range courtship behaviours may be just as important as long-range signals in mate choice and reproductive isolation and that in this species pair, parents, rather than hybrids, are the gatekeepers of gene flow.

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AUTHOR CONTRIBUTIONS

P.A.M. conceived the study and designed experiments with J.H., M.G.R. and N.W.B. Field collections were performed by P.A.M. and N.W.B. Behavioural data was collected by P.A.M., C.M. collected GC-MS data, and P.A.M., J.H. and C.M. analysed cuticular hydrocarbon profiles. P.A.M. performed all other statistical analyses with guidance from M.G.R. and N.W.B. The manuscript was written by P.A.M, M.G.R. and N.W.B.

DATA ARCHIVING

Data will be uploaded to the Dryad Digital Repository if the manuscript is accepted.

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